BY ELECTRONIC MAIL

October 23, 2017

Dr. Tala R. Henry
Director, Risk Assessment Division
Office of Pollution Prevention and Toxics
US Environmental Protection Agency
1200 Pennsylvania Avenue, N. W.
Mail Code: 7403M
Washington, DC 20460

RE: Risk Evaluation Scoping Efforts under the Toxic Substances Control Act (TSCA) for 1,4-Dioxane, Docket No. EPA-HQ-OPPT-2016-0723

Dr. Henry:

I am writing to correct an error in ACC's 1,4-Dioxane Panel September 19, 2017 submission to Docket no. EPA-HQ-OPPT-2016-0723 regarding the Agency's risk evaluation scoping efforts for 1,4-dioxane. We have found a minor calculation error in our submission of the quantitative weight of evidence (WOE) evaluation of the mutagenic mode of action for 1,4-dioxane. The WOE confidence score for the mutagenic MOA should be -45, not -49 as indicated in our original submission. The revised mutagenic MOA confidence score (-45) does not change the overall comparative MOA analysis. The comparative MOA analysis continues to demonstrate that it is highly unlikely that 1,4-dioxane induces rodent liver tumors via a mutagenic MOA, and that cytotoxicity and regenerative cellular proliferation (with a MOA score of +57) is the likely operative MOA for induction of liver tumors in rodents by 1,4-dioxane.

We kindly ask that you replace the copy of the MOA analysis currently in the docket with the enclosed, updated document which includes the correction in the confidence score. Thank you for noting this minor correction and please do not hesitate to contact me if you have any additional questions or clarifications on the quantitative MOA scoring method.

Sincerely,

Steve Risotto

Stephen P. Risotto
Senior Director
Chemical Products and Technology Division

Enclosure

October 20, 2017

IDENTIFYING THE LIKELY OPERATIVE MODE OF ACTION FOR 1,4-DIOXANE INDUCED RODENT LIVER TUMORS

Scientific Causality Confidence Scores for Potential Modes of Action: Comparing the Weight of Evidence for a Mutagenic Mode of Action to a Threshold Cytotoxicity Mode of Action for Rodent Liver Tumors

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Note: this document is an update of the September 19, 2017 case example. This update corrects a minor calculation error that was discovered in the September 19, 2017 document in which the confidence score for the mutagenic MOA was reported as -49. The correct calculated MOA score is -45, and this correction has been inserted in this revision. This minor correction in the mutagenic MOA confidence score does not change the overall comparative MOA analysis.

I. Executive Summary

Background: One of the critical elements of a chemical carcinogenic risk assessment is the determination of the likely operative mode of action (MOA). Determining the operative MOA by which a chemical can cause cancer is important for characterizing potential human health hazards and for selecting dose-response extrapolation methods for use in risk assessment at environmental levels of exposure. This case example focused on evaluating hypothesized MOAs involved in the induction of liver tumors in rodents by 1,4-dioxane.

This case example used the quantitative MOA WOE confidence scoring approach described in Becker et al, 2017[1]. This method provides a systematic and explicit approach for 1) evaluating a chemical dataset using hypothesized MOAs and the evolved Bradford Hill causal considerations (biological plausibility, essentiality, dose-response concordance, consistency, and analogy) and 2) deriving an overall confidence score for each hypothesized MOA. This enables a side-by-side comparison of numerical WOE confidence scores for each MOA, and the determination of which MOA is more likely to be operative.

Analysis and Results: Using the quantitative MOA WOE confidence scoring approach described in Becker et al, 2017, we compared the WOE for a mutagenic MOA to the WOE for a threshold cytotoxicity MOA. We summarized the relevant dose response and incidence data and developed WOE confidence scores for both a mutagenic MOA and a threshold cytotoxicity MOA (see sections III and IV for details).

This analysis indicates:

- The scientific confidence WOE score derived for induction of rodent liver tumors for a mutagenic MOA was -45. This negative WOE score indicates there is strong counter evidence for several of the early, most diagnostic KEs, for a mutagenic MOA. In other words, the available data indicate that it is highly unlikely that rodent liver tumors are induced by 1,4-dioxane acting via a mutagenic mode of action.

- The scientific confidence WOE score derived for induction of rodent liver tumors for a cytotoxic MOA was +57. Thus, the likely operative MOA for induction of liver tumors in rodents by 1,4-dioxane is cytotoxicity, which would only be operative at doses that exceed the threshold.

Conclusions and Recommendations: Based on the weight of the evidence (indicated by comparison of the MOA confidence scores), the likely operative MOA for induction of liver tumors in rodents by 1,4-dioxane is cytotoxicity, not mutagenicity. The overall pattern of observations is very consistent with a non-linear, threshold mode of carcinogenic action. Therefore, it would be inappropriate to use a linear default for extrapolating cancer risks. Instead, the MOA WOE scientific confidence scoring analysis supports use of a threshold, non-linear method for determining potential cancer risks (i.e., an extrapolation method based upon cytotoxicity, for which cancer risk would only be operative at doses that exceed the threshold for induction of hepatic cytotoxicity). As the Agency moves forward with its problem formulation and risk assessment of 1,4-dioxane, we encourage EPA to consider use of the quantitative confidence scoring method for determining and communicating the likely operative MOA based on the weight of scientific evidence and best available science. The quantitative MOA WOE confidence scoring approach is particularly useful in providing the scientific basis for justifying the selection of the most appropriate extrapolation method for determining potential human health risks. In fact, this approach should prove to be useful for most, if not all, of the TSCA assessments now being developed by OPPT that deal with potential carcinogenic risks, especially where alternative MOAs with supporting mechanistic data are credible.

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[1] Becker RA et al. 2017. Quantitative weight of evidence to assess confidence in potential modes of action. Regul Toxicol Pharmacol. 86: 205-220. OPEN ACCESS: http://www.sciencedirect.com/science/article/pii/S0273230017300387?via%3Dihub

II. Introduction

One of the critical elements of a chemical carcinogenic risk assessment is the determination of the likely operative mode of action (MOA). Understanding a chemical's mode of carcinogenic action forms the scientific basis for the selection of the dose-response extrapolation method that best aligns with the underlying biology of the specific MOA pathway, and subsequently, ensures that the best available science is used for quantifying potential cancer risks at environmental levels of exposures.

To improve transparency and objectivity in MOA analysis, the WHO/IPCS MOA framework has recently been extended using an approach that enables quantitative scoring of the confidence in the weight of the evidence of alternative hypothesized MOAs. We have attached the abstract and link to the open access, peer-reviewed publication detailing this quantitative method (Appendix B. Becker *et al.*, 2017). As described in the publication, to integrate evidence and derive confidence scores for potentially relevant MOAs, a systematic and explicit approach is used for evaluating a chemical dataset using key events in the context of the evolved Bradford Hill causal considerations. This enables a side-by-side comparison using numerical scores of scientific confidence in each hypothesized MOA, including a default mutagenic MOA, to better identify the more likely (i.e., best supported) operative MOA. We are in the process of developing several additional case examples on a variety of chemicals to further illustrate the application of the MOA quantitative confidence scoring method and to support the continued refinement of the method.

As EPA commences the risk assessment of 1,4-dioxane we believe the application of the quantitative MOA confidence scoring method can, as part of EPA's consideration of best available science and weight of the evidence, inform the Agency's problem formulation phase of the cancer risk assessment.

The quantitative MOA WOE confidence scoring approach detailed in Becker et al., 2017 is a systematic and explicit approach for evaluating a chemical dataset, using hypothesized MOAs and the evolved Bradford Hill causal considerations (biological plausibility, essentiality, dose-response concordance, consistency, and analogy), to integrate evidence and derive confidence scores for potentially relevant MOAs. The components consist of: 1) a set of defining questions for each of the Bradford Hill considerations coupled with a WOE rating and scoring procedure to guide data evaluation and WOE determinations; 2) a procedure to score the evolved Bradford Hill causal consideration for essentiality at MOA pathway level based on the highest score achieved by any one of the unique Key Events (KEs) in the pathway; 3) a technique for including the supporting evidence of later KEs, even though these are disease-specific and not diagnostic of a MOA for a particular chemical, by affording less evidentiary value to later KEs than the earlier, more MOA-specific KEs; 4) hierarchical weighting of the evolved Bradford Hill causality considerations; and 5) a straightforward arithmetic method to characterize the overall confidence score for each hypothesized MOA.

To document the application of the recently peer-reviewed quantitative MOA WOE confidence scoring approach (Becker et al., 2017), we have developed this case example using the published 1,4-dioxane rodent liver tumor MOA data. The steps of quantitative MOA WOE confidence scoring are discussed in detail in Becker et al., 2017; they are briefly described here to help contextualize the case example tables presented in Sections III and IV.

- Step 1. Identification of postulated MOAs and KEs/KERs for the adverse outcome(s) of interest (See Section 2.1 of Becker et al. 2017). (Note: Steps 2 through 5 are conducted for each hypothesized MOA.)

- Step 2. Qualitatively evaluate the evidence in support of, or inconsistent with, the KEs/KERs (See Section 2.2 of Becker et al. 2017) using the evolved Bradford Hill causal considerations.

- Step 3. Quantitatively rate each KE/KER using the evolved Bradford Hill causal considerations (See Section 2.3.1 of Becker et al. 2017). In the qualitative and quantitative rating approach (Steps 2 and 3), the individual or series of KEs are evaluated against the defining question for each evolved Bradford Hill causal consideration, using the WOE rating categories to guide the determinations for scoring. The rating categories include strong (3), moderate (2), weak (1), no evidence (0), weak counter evidence (-1), moderate counter evidence (-2), and strong-counter evidence (-3)).

- Step 4. Derive the composite score for each KE/KER by multiplying the quantitative rating score by the weight assigned for each of the evolved Bradford Hill causal considerations and adjust based on the MOA evidentiary value of each KE/KER ($\sum$ (weight $\times$ rate $\times$ evidentiary value) = KE/KER score) (See Section 2.3.2 of Becker et al. 2017). To derive the composite score, each Bradford Hill causal consideration has been given a numerical weight in accordance with their ranked importance, with a summed maximum of 100% (Section 2.3.1 of Becker et al, 2017). Essentiality of the KEs within the MOA is considered collectively since the interdependence of KEs is often illustrated through the impact of prevention or augmentation of an earlier KE on later KEs. Furthermore, all KEs/KERs are not necessarily weighted the same. This is because for a given adverse outcome, often the later KEs leading to the adverse outcome are the same for each of the hypothesized MOAs. These later KEs are often indicative of the disease process, whereas the earlier KEs are more chemical-specific, so in this method the later KES that are common across MOAs are assigned an evidentiary weighting value of 10%.
- Step 5. Integrate the evidence of causality for the MOA by calculating the sum of the scores for all KEs/KERs and then dividing by the total maximum score to derive the "MOA confidence score" (See Section 2.4 of Becker et al. 2017). To calculate the overall WOE confidence score for a hypothesized MOA, KE scores are summed and normalized by dividing by the maximum possible score and then multiplying by 100. This simple normalization procedure allows for comparison of quantitative confidence scores in cases where the number of KEs differs between hypothesized MOAs. Total scores may be negative if, for a hypothesized MOA, there is strong counter evidence for several of the early, most diagnostic KEs.

- Step 6. Compare the quantitative confidence scores for the hypothesized MOAs, and select the MOA for which confidence in the supporting data is highest (See Section 2.4 of Becker et al. 2017).

Using the quantitative MOA WOE confidence scoring approach, in Sections III and IV below, we have summarized the relevant dose response and incidence data and developed WOE confidence scores for a mutagenic MOA and a threshold cytotoxicity MOA.

Note: The intent of this case study is to illustrate the quantitative scoring methodology. It is not intended to be a complete discussion of all available and relevant studies. To that end, we did not conduct an in-depth systematic review of the available literature, but we based this evaluation on data and lines of evidence from already published review articles, and those authors' evaluations of the quality of the empirical evidence. The data and lines of evidence used in developing this case example are from scientifically peer reviewed and journal published articles. As indicated in Becker et al., 2017, with respect to biological plausibility of hypothesized modes of action leading to cancer, both the mutagenic mode of action and the cytotoxic/regenerative cell proliferation mode of action are considered to be well documented, and thus the biological plausibility of these MOAs are not considered further herein. Rather, chemical-specific supporting data for the evolved Bradford Hill causal considerations (essentiality, dose, incidence and temporal concordance, consistency and analogy) are specifically evaluated for the Key Events/Key Event Relationships (KEs/KERs) as a basis to apply the quantitative weight of evidence confidence scoring method.

Acknowledgments: This project was partly supported by contributions from ACC's Science and Research Division and from ACC's Center for Advancing Risk Assessment Science and Policy.

III. Evaluating the WOE for a Mutagenic MOA

MUTAGENIC MODE OF ACTION

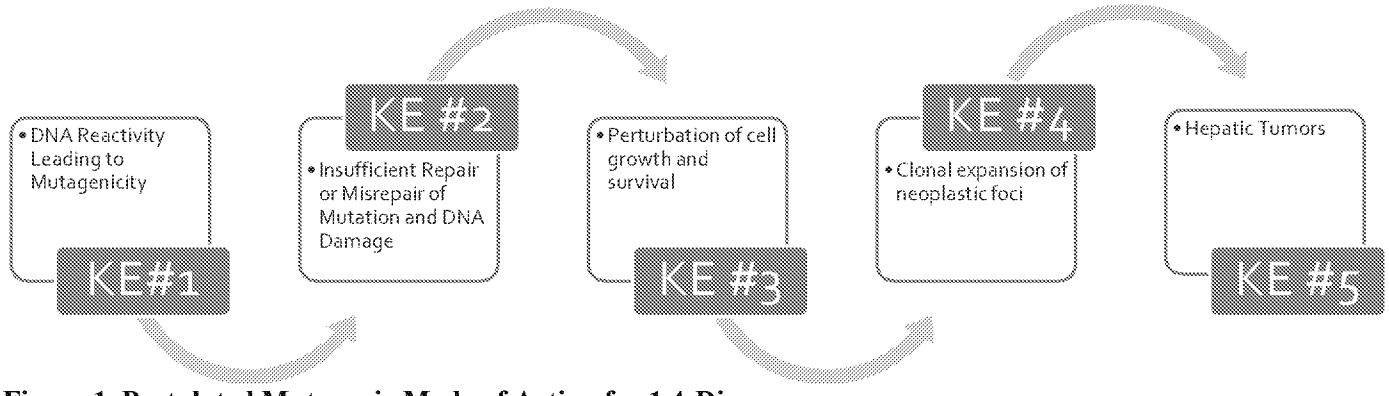


Figure 1. Postulated Mutagenic Mode of Action for 1,4-Dioxane

The genotoxicity data for 1,4-Dioxane for *in vitro* and *in vivo* tests are robust. 1,4-Dioxane has been tested for genotoxic potential using *in vitro* assay systems with prokaryotic organisms, non-mammalian eukaryotic organisms, and mammalian cells, and *in vivo* assay systems using several strains of rats and mice. In the large majority of *in vitro* systems, 1,4-dioxane was not genotoxic. Where positive genotoxic response was observed, it was generally in the presence of cytotoxicity. More importantly, 1,4-dioxane was not genotoxic in the majority of *in vivo* studies. 1,4-Dioxane did not bind covalently to DNA with calf thymus DNA. Several investigators have reported that 1,4-dioxane caused increased DNA synthesis indicative of cellular proliferation. Overall, the available literature clearly indicates that 1,4-dioxane is non-genotoxic or at best, weakly genotoxic.

A. Qualitative Evaluation of the Weight of Evidence (WOE) for 1,4-Dioxane Acting via a Mutagenic MOA

Table 1. Qualitatively Evaluate the Comparative Weight of Evidence for 1,4 Dioxane Acting via the Mutagenic MOA (USEPA, 2010) – (see Appendix I. for additional study summaries)

Key Event	Supporting Data	Potentially Inconsistent	References
KE#1 DNA reactivity leading to mutagenicity	Morita and Hayashi (1998,) demonstrated an increase in micronucleus (MN) formation in hepatocytes following 1,4-dioxane dosing and partial hepatectomy to induce cellular mitosis [MN formation is an indication of chromosomal damage, which is not the same as a gene mutation; therefore the results are not considered definitive for identifying a mutational effect]. DNA single-strand breaks were demonstrated in hepatocytes following gavage exposure to female rats (Kitchin and Brown, 1990). At high doses 1,4-dioxane exerts	Negative findings were reported for mutagenicity in *in vitro* assays with the prokaryotic organisms *Salmonella typhimurium*, *Escherichia coli*, and *Photo bacterium phosphoreum* (Mutatox assay) (Haworth et al., 1983; Hellmér and Bolcsfoldi, 1992; Khudoley et al., 1987; Kwan et al., 1990; Morita and Hayashi, 1998; Nestmann et al., 1984; Stott et al., 1981). In *in vitro* assays with nonmammalian eukaryotic	(Haworth et al., 1983; Hellmér and Bolcsfoldi, 1992; Khudoley et al., 1987; Kwan et al., 1990; Morita and Hayashi, 1998; Nestmann et al., 1984; Stott et al., 1981. Yoon et al., 1985; Zimmermann et al., 1985 Munoz and Barnett, 2002; Kitchin and Brown, 1990; Morita and Hayashi (1998)

Key Event	Supporting Data	Potentially Inconsistent	References
	genotoxic effects in both the mouse bone marrow and liver.	organisms, negative results were obtained for the induction of aneuploidy in yeast (*Saccharomyces cerevisiae*) and in the sex-linked recessive lethal test in Drosophila melanogaster (Yoon et al., 1985; Zimmermann et al., 1985. In the presence of toxicity, positive results were reported for meiotic nondisjunction in Drosophila (Munoz and Barnett, 2002).	
KE#2 Insufficient repair	No data providing evidence of insufficient repair or misrepair of mutation and DNA damage	1,4-Dioxane did not affect *in vitro* or *in vivo* DNA repair in hepatocytes or *in vivo* DNA repair in the nasal cavity (Goldsworthy et al., 1991; Stott et al., 1981), but increased hepatocyte DNA synthesis indicative of cell proliferation in several *in vivo* studies (Goldsworthy et al., 1991; Miyagawa et al., 1999; Stott et al., 1981; Uno et al., 1994).	
KE#3 Perturbation of cell growth and survival	Liver effects reported to include hepatocyte degeneration and necrosis, hepatocyte swelling.		Argus et al., 1965; Argus et al., 1973; Fairley et al., 1934; Kano et al., 2008; Kociba et al., 1974; NCI, 1978
KE#4 Clonal expansion of neoplastic foci	Liver hepatocyte hyperplasia and clear and mixed foci development in the liver were reported. Spongiosis hepatitis, hyperplasia, and clear and mixed-cell foci were also observed in the liver of rats (doses >55 mg/kg-day in male rats) (JBRC, 1998, 196240; Kano et al., 2009, 594539)		JBRC, 1998; Kano et al., 2009
KE#5 Liver Tumors	Findings from JBRC (1998, 196240) also provided evidence of liver hyperplasia in male F344/DuCrj rats at a dose level		JBRC, 1998; Kano et al., 2009 Kociba et al., 1974; NCI, 1978; Yamazaki et al., 1994.

Key Event	Supporting Data	Potentially Inconsistent	References
	below the dose that induced a statistically significant increase in tumor formation.		

KE#1 DNA reactivity leading to Mutagenicity (Extracted from IRIS Assessment for 1,4-Dioxane – USEPA 2010)

Supporting Data

Morita and Hayashi (1998) demonstrated an increase in micronucleus formation in hepatocytes following 1,4-dioxane dosing and partial hepatectomy to induce cellular mitosis. DNA single-strand breaks were demonstrated in hepatocytes following gavage exposure to female rats (Kitchin and Brown, 1990). Mirkova (1994), reported a dose-related increase in the incidence of bone marrow micronuclei in male and female C57BL6 mice 24 or 48 hours after administration of 1,4-dioxane. At a sampling time of 24 hours, a dose of 450 mg/kg produced no change relative to control, while doses of 900, 1,800, and 3,600 mg/kg increased the incidence of bone marrow micronuclei by approximately two-, three-, and fourfold, respectively. As noted above, these resulst were not replicated in an independent study by Tinwell and Ashby (1994) using the same mouse strain (C57BL6).

Roy et al. (2005) examined micronucleus formation in male CD1 mice exposed to 1,4-dioxane to confirm the mixed findings from earlier mouse micronucleus studies and to identify the origin of the induced micronuclei. Mice were administered 1,4-dioxane by gavage at doses of 0, 1,500, 2,500, and 3,500 mg/kg-day for 5 days. The mice were also implanted with 5-bromo-2-deoxyuridine (BrdU)-releasing osmotic pumps to measure cell proliferation in the liver and to increase the sensitivity of the hepatocyte assay. The frequency of micronuclei in the bone marrow erythrocytes and in the proliferating BrdU-labeled hepatocytes was determined 24 hours after the final dose. Significant dose-related increases in micronuclei were seen in the bone marrow at all the tested doses (≥ 1,500 mg/kg-day). In the high-dose (3,500-mg/kg) mice, the frequency of bone marrow erythrocyte micronuclei was about 10-fold greater than the control frequency. Significant dose-related increases in micronuclei were also observed at the two highest doses (≥ 2,500 mg/kg-day) in the liver. Antikinetochore (CREST) staining or pancentromeric fluorescence in situ hybridization (FISH) was used to determine the origin of the induced micronuclei. The investigators determined that 80–90% of the micronuclei in both tissues originated from chromosomal breakage; small increase in micronuclei originating from chromosome loss was seen in hepatocytes. Dose-related statistically significant decreases in the ratio of bone marrow polychromatic erythrocytes (PCE):normochromatic erythrocytes (NCE), an indirect measure of bone marrow toxicity, were observed. Decreases in hepatocyte proliferation were also observed.

Based on these results, the authors concluded that at high doses 1,4-dioxane exerts genotoxic effects in both the mouse bone marrow and liver; the induced micronuclei are formed primarily from chromosomal breakage; and 1,4-dioxane can interfere with cell proliferation in both the liver and bone marrow. The authors noted that reasons for the discrepant micronucleus assay results among various investigators was unclear, but could be related to the inherent variability present when detecting moderate to weak responses using small numbers of animals, as well as differences in strain, dosing regimen, or scoring criteria.

Potentially Inconsistent Data

1,4-Dioxane did not covalently bind to DNA under *in vitro* study conditions (Woo et al., 1977). DNA alkylation was not detected in the liver 4 hours following single gavage exposure (1000 mg/kg) in male Sprague Dawley rats (Stott et al., 1981, 063021).

Negative findings were reported for mutagenicity in *in vitro* assays with the prokaryotic organisms *Salmonella typhimurium*, *Escherichia coli*, and *Photobacterium phosphoreum* (Mutatox assay) (Haworth et al., 1983; Hellmér and Bolcsfoldi, 1992; Khudoley et al., 1987; Kwan et al., 1990; Morita and Hayashi, 1998; Nestmann et al., 1984; Stott et al., 1981). In *in vitro* assays with nonmammalian eukaryotic organisms, negative results were obtained for the induction of aneuploidy in yeast (*Saccharomyces cerevisiae*) and in the sex-linked recessive lethal test in Drosophila melanogaster (Yoon et al., 1985; Zimmermann et al., 1985). In the presence of toxicity, positive results were reported for meiotic nondisjunction in Drosophila (Munoz and Barnett, 2002).

The ability of 1,4-dioxane to induce genotoxic effects in mammalian cells *in vitro* has been examined in model test systems with and without exogenous metabolic activation and in hepatocytes that retain their xenobiotic-metabolizing capabilities. 1,4-Dioxane was reported as negative in the mouse lymphoma cell forward mutation assay (McGregor et al., 1991; Morita and Hayashi, 1998). 1,4-Dioxane did not produce chromosomal aberrations or micronucleus formation in Chinese hamster ovary (CHO) cells (Galloway et al., 1987; Morita and Hayashi, 1998). Results were negative in one assay for sister chromatid exchange (SCE) in CHO (Morita and Hayashi, 1998) and were weakly positive in the absence of metabolic activation in another (Galloway et al., 1987). In rat hepatocytes, 1,4-dioxane exposure *in vitro* caused single-strand breaks in DNA at concentrations also toxic to the hepatocytes (Sina et al., 1983) and produced a positive genotoxic response in a cell transformation assay with BALB/3T3 cells also in the presence of toxicity (Sheu et al., 1988).

1,4-Dioxane was not genotoxic in the majority of available *in vivo* mammalian assays. Studies of micronucleus formation following *in vivo* exposure to 1,4-dioxane produced mostly negative results, including studies of bone marrow micronucleus formation in B6C3F1, BALB/c, CBA, and C57BL6 mice (McFee et al., 1994; Mirkova, 1994,; Tinwell and Ashby, 1994) and micronucleus formation in peripheral blood of CD1 mice (Morita, 1994; Morita and Hayashi, 1998). One study, Mirkova (1994), reported a dose-related increase in the incidence of bone marrow micronuclei in male and female C57BL6 mice 24 or 48 hours after administration of 1,4-dioxane. At a sampling time of 24 hours, a dose of 450 mg/kg produced no change relative to control, while doses of 900, 1,800, and 3,600 mg/kg increased the incidence of bone marrow micronuclei by approximately two-, three-, and fourfold, respectively. A dose of 5,000 mg/kg also increased the incidence of micronuclei by approximately fourfold at 48 hours. This compares with the negative results for BALB/c male mice tested in the same study at a dose of 5,000 mg/kg and sampling time of 24 hours. However, Tinwell and Ashby (1994, 195086) could not explain the difference in response in the mouse bone marrow micronucleus assay with C57BL6 mice obtained in their laboratory (i.e., non-significant 1.6-fold increase over control) with the dose-related positive findings reported by Mirkova (Mirkova, 1994, 195062) using the same mouse strain, 1,4-dioxane dose (3,600 mg/kg) and sampling time (24 hours). Thus, although supportive of a potential genotoxic effect, the Mirkova (1994) study results are considered inconsistent as the results were not replicated or consistently observed by other investigators. Furthermore, MN formation is an indication of chromosomal damage, which is not the same as a gene mutation; therefore the results are not considered definitive for identifying a mutational effect.

KE#2 Insufficient Repair of Misrepair of Mutation and DNA Damage

Supportive Data

Not available

Potentially Inconsistent Data

1,4-Dioxane did not affect *in vitro* or *in vivo* DNA repair in hepatocytes or *in vivo* DNA repair in the nasal cavity (Goldsworthy et al., 1991; Stott et al., 1981), but increased hepatocyte DNA synthesis indicative of cell proliferation in several *in vivo* studies (Goldsworthy et al., 1991; Miyagawa et al., 1999; Stott et al., 1981; Uno et al., 1994).

KE#3 Perturbation of Cell Growth and Survival

Supportive Data

Liver effects included degeneration and necrosis, hepatocyte swelling, cells with hyperchromic nuclei, spongiosis hepatis, hyperplasia, and clear and mixed cell foci of the liver (Argus et al., 1965; Argus et al., 1973; Fairley et al., 1934; Kano et al., 2008; Kociba et al., 1974; NCI, 1978). Hepatocellular degeneration and necrosis were seen at high doses in a subchronic study (1,900 mg/kg-day in rats) (Fairley et al., 1934) and at lower doses in a chronic study (94 mg/kg-day, male rats) (Kociba et al., 1974). Argus et al. (1973) described a progression of preneoplastic effects in the liver of rats exposed to a dose of 575 mg/kg-day.

Potentially Inconsistent Data

Not available

KE #4 Clonal Expansion of Neoplastic Foci

Supportive Data

Kociba et al. (1974) noted evidence of liver toxicity at or below the dose levels that produced liver tumors but did not report incidence data for these effects. Hepatocellular degeneration and necrosis were observed in the mid- and high-dose groups of male and female Sherman rats exposed to 1,4-dioxane, while tumors were only observed at the highest dose. Hepatic regeneration was indicated in the mid- and high-dose groups by the formation of hepatocellular hyperplastic nodules. Findings from JBRC (1998) also provided evidence of liver hyperplasia in male F344/DuCrj rats at a dose level below the dose that induced a statistically significant increase in tumor formation.

Potentially Inconsistent Data

Not available

KE #5 Liver Tumor Formation

Supportive Data

Liver tumors have been observed following drinking water exposure in male Wistar rats (Argus et al., 1965,), male guinea pigs (Hoch-Ligeti and Argus, 1970,), male Sprague Dawley rats (Argus et al., 1973; Hoch-Ligeti et al., 1970,), male and female Sherman rats (Kociba et al., 1974, 0), female Osborne-Mendel rats (NCI, 1978,), male and female F344/DuCrj rats (JBRC, 1998,; Kano et al., 2009,; Yamazaki et al., 1994,), male and female B6C3F1 mice (NCI, 1978,), and male and female Crj:BDF1 mice (JBRC, 1998; Kano et al., 2009, 594539; Yamazaki et al., 1994). In the earliest cancer bioassays, the liver tumors were described as hepatomas (Argus et al., 1965; Argus et al., 1973; Hoch-Ligeti and Argus, 1970,; Hoch-Ligeti et al., 1970, however, later studies made a distinction between hepatocellular carcinoma and hepatocellular adenoma (JBRC, 1998,; Kano et al., 2009; Kociba et al., 1974,; NCI, 1978; Yamazaki et al., 1994). Both tumor types have been seen in rats and mice exposed to 1,4-dioxane.

Potentially Inconsistent Data

Not available

Table 2. Dose and Temporal Concordance Table for the Mutagenic MOA.

[For details on dose levels and responses see Dourson et al. 2014 and Dourson et al., 2017]

Temporal Concordance →

Dose Concordance ↓

Temporal / Dose/Conc.	<24hrs. DNA Reactivity	1-2 weeks Insufficient Repair	1-2 weeks Perturbation of growth	2-yrs. Clonal Expansion	2 yrs. Liver Tumors
1500 mg/kg bw	--- (did not bind to DNA and overall negative in vitro and in vivo assays)	--- (did not affect DNA repair – Goldworthy 1991)	-- (did not affect cell proliferation – Nannelli et al. 2005)	No Data	No Data
1168 mg/kg-bw (adjusted chronic dose 389 mg/kg-day) or 2year doses of 55 mg/kg bw-day	No Data	No Data	++	+++	No Data
274 mg/kg bw-day in 2 year study	No Data	No Data	No Data	No Data	+++

+++ = strong evidence ++ = moderate evidence + = weak evidence
--- strong counter evidence -- = moderate counter evidence - = weak counter evidence

B. Evolving Bradford Hill Causal Considerations: Qualitative Data Evaluation

Table 3. Evolved Bradford Hill causal considerations, defining questions and body of evidence (adapted from Meek et al., 2014 a,b).

Bradford Hill Causal Considerations	Defining questions	Supporting Evidence	Potentially inconsistent Evidence	Weight of Evidence Scoring
Biological Plausibility	Does the hypothesized MOA conflict with broader knowledge? How well established is the MOA?	MOA is well established in scientific knowledge and consistent with biological understanding.		Not scored
Essentiality	Is the sequence of events reversible if dosing is stopped or a KE is prevented?	No evidence	No evidence	0

Empirical Support – Dose and Incidence Concordance	Dose concordance: Are the KEs observed at doses below or similar to those associated with the adverse effect? Incidence concordance: Is the occurrence of the adverse effect (e.g., hepatocellular carcinoma) less than that for the preceding KEs?		Weight of evidence does not support a mutagenic mode of action based on the inability to interact with DNA, interfere with repair mechanisms and perturb cell growth and survival.	-3 (strong counter evidence)
Empirical Support – Temporal Concordance	Temporal concordance: Are the KEs observed in hypothesized order?		No indications of initiating the mutagenic mode of action pathway	-3 (strong counter evidence)
Consistency	What is the pattern of observations across species/strains/organs/test systems? What would be expected based on the hypothesized MOA?		*In vitro* and *in vivo* genotoxicity data indicate negative mutagenic mode of action. Later key events are part of the normal biological progression for liver tumors and not indicative of a mutagenic mode of action.	-3 (strong counter evidence)
Analogy	Would the MOA be anticipated based on broader chemical specific knowledge (e.g., the chemical is a member of a category for which related chemicals have known or strongly suspected MOA)?		A mutagenic mode of action would be inconsistent with other solvents that act via a nongenotoxic mode of action.	-3 (strong counterevidence)

C. Qualitative and Quantitative Rating of the Key Events for Bradford Hill Causal Considerations

Qualitative and quantitative rating categories. [See Becker et al. 2017 for details]

Qualitative	Quantitative	Category description
Strong	3	Multiple studies and/or extensive data provide convincing evidence that the substance causes the KE.
Moderate	2	Some evidence (direct or indirect) indicating the substance causes the KE, but scientific understanding is not yet completely established. There may be some studies that are equivocal.
Weak	1	Very limited evidence (direct or indirect) that the substance causes the KE along this pathway. Scientific understanding of the KE is limited.
No Evidence	0	No data available to support or negate causation of this KE by the substance.
Weak Counter	−1	There is very limited contradictory evidence (direct or indirect) that the substance does not cause this KE.
Moderate Counter	−2	Some evidence (direct or indirect) indicating that the KE is not caused by the substance, but scientific understanding is not completely established. There may be some studies that are equivocal.
Strong Counter	−3	Multiple studies and/or extensive data provide convincing evidence that the substance does not cause this KE.

Table 4. Qualitative Rating of the Key Events for Bradford Hill Causal Considerations (Step 3)

Bradford Hill Causal Considerations	Key Event #1	Key Event #2	Key Event #3	Key Event #4	Key Event #5
	DNA reactivity	Insufficient repair	Perturbation of cell growth and survival	Clonal expansion of pre-neoplastic foci	Liver tumors
Biological Plausibility	Not scored in this version				
Essentiality	0	0	0	0	0
Empirical Support – Dose and Incidence Concordance	-3	-3	2	3	3
Empirical Support – Temporal Concordance	-3	-3	2	3	3
Consistency	-3	-3	2	3	3
Analogy	-3	-3	2	3	3

D. Quantification of the WOE for a Mutagenic MOA

Table 5. Quantification of the WOE for the mutagenic MOA (Step 4 and 5).
[See Becker et al. 2017 for details on weighting the BH considerations and early vs. common later KES - each evolved Bradford Hill causal consideration has been given a numerical weight in accordance with their ranked importance, with a summed maximum of 100% and the later KEs are afforded 10% of the probative value of the early KEs]. Score = rating x weight.

Bradford Hill Causal Considerations	Key Event #1	Key Event #2	Key Event #3	Key Event #4	Key Event #5
	DNA reactivity	Insufficient repair	Perturbation of cell growth and survival	Clonal expansion of pre-neoplastic foci	Liver tumors
Essentiality (40%)	0	0	0	0	0
Empirical Support – (20%) Dose and Incidence Concordance	-3 (.2) = - 0.6	-3 (.2) = -0.6	2 (.2) = 0.4	3 (.2) = 0.6	3(.2) = 0.6
Empirical Support – (20%) Temporal Concordance	-3 (.2) = - 0.6	-3 (.2) = -0.6	2 (.2) = 0.4	3 (.2) = 0.6	3 (.2) = 0.6
Consistency (10%)	-3(.1) = - 0.3	-3 (.1) = -0.3	2 (.1) = 0.2	3 (.1) = 0.3	3 (.1) = 0.3
Analogy (10%)	-3 (.1) = - 0.3	-3 (.1) = -0.3	2 (.1) = 0.2	3 (.1) = 0.3	3 (.1) = 0.3
TOTAL	-1.8	-1.8	1.2 (0.1)	1.8 (0.1)	1.8 (0.1)

Mode of Action Confidence Score = -3.36/6.9 = -45 which indicates a low likelihood of this MOA being operative for 1,4-dioxane.

Narrative Characterization Comparing the MOA Confidence Scores for the Hypothesized Modes of Action – (Step 6)

A mutagenic mode of action for 1,4-dioxane is not supported by the available data. The database does not demonstrate that 1,4-dioxane has an ability to initiate the mode of action via interaction with DNA to elicit mutagenicity. Available studies do not provide evidence that exposure to 1,4-dioxane is associated with reaction of the parent compound or metabolite with DNA. Evidence is also lacking of effects of 1.4-dioxane on insufficient/ misrepair of promutagenic DNA damage. Some studies reported chromosomal effects (micronuclei formation) but these appear to occur at high (cytotoxic) doses or were not reproduced in independent studies. Remaining downstream key events are part of the normal biological progression towards liver tumors and are not diagnostic of a mutagenic mode of action. In comparing the quantitative mode of action scores between the cytotoxic regenerative proliferation mode of action (+57) to the score for mutagenic mode of action (-45), the magnitude of difference support the more likely mode of action for 1,4-dioxane to be nongenotoxic and requiring sustained regenerative proliferation.

E. Key References

Becker RA, Dellarco VD, Seed J, Kronenberg JM, Meek B, Foreman J, Palermo C, Kirman C, Linkov I, Schoeny R, Dourson M, Pottenger LH, Manibusan MK. 2017. Quantitative Weight of Evidence to Assess Confidence in Potential Modes of Action. Regulatory Toxicology and Pharmacology. 86(2017) 205-220.

Dourson M, Reichard J, Nance P, and McConnell E. 2014. Mode of Action Analysis for Liver Tumors from Oral 1,4-Dioxane Exposures and Evidence-Based Response Assessment.

USEPA. 2010. Toxicological Review of 1,4-Dioxane (CAS No. 123-91-1) In Support of Summary Information on the Integrated Risk Information System (IRIS). August 2010. EPA/635/R-09/005/F. www.epa.gov/iris.

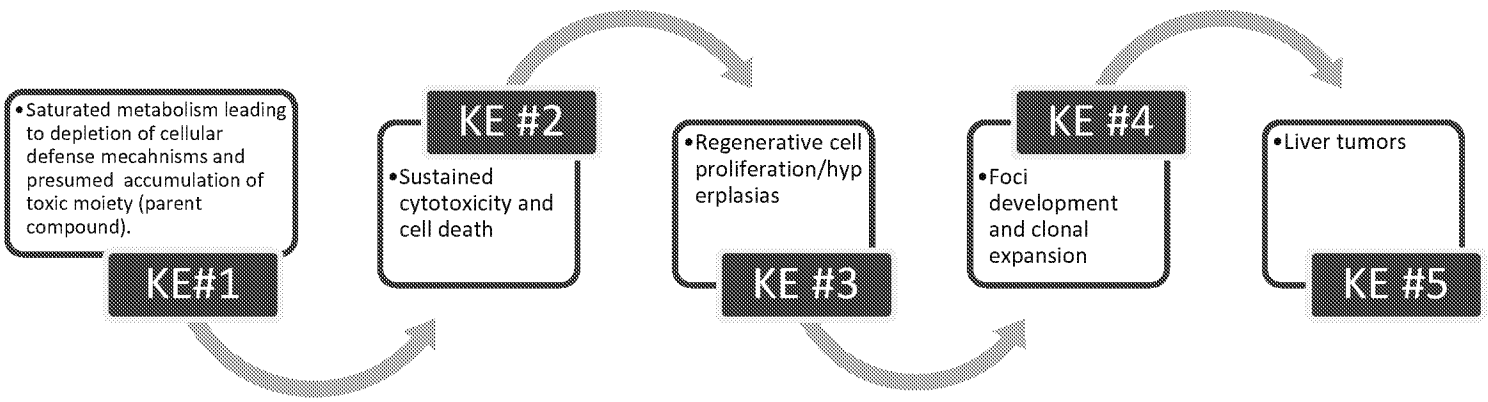
IV. Evaluation the WOE for a Cytotoxic Regenerative Cell Proliferation MOA

The cytotoxic regenerative cell proliferation MOA pathway for 1,4-dioxane is illustrated in Figure 2. In this MOA, hepatic cytotoxicity leads to an increase in regenerative cell proliferation. Regenerative proliferation increases the number of DNA replications causing an increase in spontaneous errors in DNA. This can lead to development of preneoplastic lesions and these altered cells may eventually progress to produce carcinomas. For this MOA, cytotoxicity is a threshold phenomenon, and since it is a critical key event, carcinogenicity is also a threshold phenomenon within this MOA.

The cytotoxic MOA in rats involves an initial key event (KE#1) that includes saturated metabolism leading to depletion of cellular defenses and presumed accumulation of proximal toxic moiety (parent compound). Subsequent sustained cytotoxicity and cellular necrosis (KE#2) are observed. Necrosis and/or inflammation, is shown to occur in a subchronic 13-week study with animals administered doses as low as 657 mg/kg-day (chronic dose equivalent[2] of 219 mg/kg-day), or 2-year doses as low as 94 mg/kg-day. Increased DNA synthesis (KE#3) as measured by [3H]-thymidine incorporation, is shown to occur in an 11-week study with administered doses as low as 1000 mg/kg-day (chronic dose equivalent of 330 mg/kg-day). Hepatocellular hyperplasia (KE#3), is also shown to occur at administered 11-week doses as low as 1000 mg/kg-day (chronic dose equivalent of 330 mg/kg-day, and is seen at administered 2-year doses as low as 55 mg/kg-day. Pre-neoplastic foci (KE#4), are seen at administered 13-week doses as low as 1168 mg/kg-day (chronic dose equivalent of 389 mg/kg-day), or 2-year doses as low as 55 mg/kg-day. Finally, the apical effect, adenomas and/or carcinomas (KE#5) is not seen at 13 weeks, but does occur after two years at doses as low as 274 mg/kg-day.

In mice, the same hypothesized mode of action applies, with the same initiating event leading to hepatocellular toxicity, swelling, hypertrophy and liver weight increases, which occur at administered 13-week doses as low as 585 mg/kg-day (chronic dose equivalent of 195 mg/kg-day) or 2-year doses as low as 191 mg/kg-day. Necrosis and/or inflammation, is also shown to occur at administered 13-week doses as low as 585 mg/kg-day (chronic dose equivalent of 195 mg/kg-day), or 2-year doses as low as 191 mg/kg-day. DNA synthesis was not reported in mice. Hepatocellular hyperplasia, is not shown to occur in the 13 week study, but is also seen in the 2-year dose of 380 mg/kg-day (interestingly this effect is not recorded for the high dose of the NCI bioassay, see Dourson et al., 2017). Pre-neoplastic foci, was also not reported in the 13-week doses, but is found at administered 2-year doses as low as 380 mg/kg-day in the McConnell re-read of the NCI (1978) bioassay, but was generally not found in JBRC (1990a) nor its publication by Kano et al. (2009). Finally, the apical effects, adenomas and/or carcinomas are not seen at 13-weeks, as expected, but do occur after two years at doses between 66-964 mg/kg-day.

Figure 2. Postulated Cytotoxic Mode of Action for 1,4-Dioxane



[2] Calculations of chronic dose equivalents are described in detail in Dourson et al., 2014.

A. Qualitative Evaluation of the Weight of Evidence (WOE) for 1,4-Dioxane Acting via a Cytotoxic MOA

Table 1. Qualitatively Evaluate the Comparative Weight of Evidence for 1,4-Dioxane Acting via a Cytotoxic MOA (Dourson et al., 2014)

[Note: the dose/cellular exposure - response relationship is described in detail in Dourson et al., 2014 and Dourson et al., 2017.]

Key Event	Supporting Data	Potentially Inconsistent	References
KE#1 – Saturated metabolism leading to depletion of cellular defenses and presumed accumulation of toxic moiety (parent compound).	Exceedances in metabolic capacity result in toxicological manifestations (Argus et al., 1965, 1973; Kociba et al., 1974, 1975; NCI, 1978). A study that gave numerous doses below the level of saturated metabolism, over 13-week duration in both rats and mice, did not demonstrate non-cancer effects at these doses (Kana et al., 2008).	No data available	Argus et al., 1965, 1973; Kociba et al., 1974, 1975; NCI, 1978; Kana et al., 2008
KE#2 – Sustained cytotoxicity and necrosis	Evidence of hepatocellular damage preceding tumor formation at higher doses of 1,4-Dioxane has been reported (Kociba et al., 1971, 1974). Liver changes including single cell necrosis at doses that saturate metabolism and deplete glutathione have been reported as early as 11 weeks post 1,4-dioxane exposure (Stott et al., 1981; Kano et al., 2008; Kaisai et al., 2008, 2009). Stott et al. (1981) reported signs of liver cytotoxicity at 1000 mg/kg /day, but not at lower doses (10 mg/kg/day) following 11 weeks of exposure.	Some differences in reporting apical endpoints were noted across studies. (Dourson et al., 2017)	Kociba et al., 1971, 1974; Stott et al., 1981; Kano et al., 2008; Kaisai et al., 2008, 2009;Dourson et al., 2017.
KE#3 – Regenerative cell proliferation	Dose response and temporal data support the occurrence of cell proliferation and hyperplasia prior to the development of liver tumors in the rat model (Kociba et al., 1971, 1974). Using replicative DNA synthesis as a surrogate marker for cell proliferation, increased hepatocyte proliferation is typically reported at tumorigenic doses of 1,4 dioxane (Goldsworthy et al., 1991; Miyagawa et al., 1999; Stott et al., 1981; Uno et al., 1994)	Some differences in reporting apical endpoints were noted across studies. (Dourson et al., 2017)	Kociba et al., 1971, 1974; Goldsworthy et al., 1991; Miyagawa et al., 1999; Stott et al., 1981; Uno et al., 1994; Kano et al., 2009; Dourson et al., 2017.
KE#4 – Preneoplastic foci development and clonal expansion	Limited data provides evidence of liver foci development and clonal expansion. Study by Lundberg et al., 1987 evaluated tumor promotion ability of 1,4 dioxane after initiation and partial hepatectomy in terms of significant GGT-positive foci and lipid accumulation. Statistically increased foci volume was evident at 1000 mg/kg/day, the highest dose tested.	The Lundberg et al., 1987 study reports GGT-positive foci volume increase and lipid accumulation, but this study does not report whether other histopathological correlates were observed. Kano et al., 2009 reports hyperplasia and foci development in male rats, but no progression to liver tumors.	Lundberg et al., 1987; Kano et al., 2009.
KE#5 – Liver tumors	Liver tumors evident in both longer-term, chronic cancer bioassays in rat and mice at high doses tested (Kano et	"Histopathological characterizations of McConnell (2013) and of JBRC (1990a) in	Kano et al., 2009; NCI, 1978 and Dourson et al., 2017

Key Event	Supporting Data	Potentially Inconsistent	References
	al., 2009 and NCI, 1978).	mice do not agree. While McConnell (2013) found extensive liver noncancerous toxicity as demonstrated by histopathology and fewer tumors than JBRC (1990), JBRC reported more tumors and nearly an absence of liver noncancerous histopathology in the chronic study which is unexpected especially with an increase in liver enzymes associated with cell damage found in this same study" (Dourson et al., 2017).	

KE#1 Saturated Metabolism leading to depletion of cellular defense mechanism and presumed accumulation of toxic moiety (parent compound)

Supporting Data – Strong (+3)

Exceedance of metabolic capacity, leading to depletion of cellular defense mechanism and presumed accumulation of toxic moiety (parent compound) occurs in association with cytotoxicity. As an initial key event, the expectation is a non-linear dose response on the basis that saturation of metabolic capacity and other cellular defenses has to achieve a pragmatic threshold for triggering a biologically relevant response. Data from humans, rats and mice demonstrate the ability to extensively metabolize 1,4-Dioxane. As doses of 1,4-dioxane increase, a transition occurs from linear first order pharmacokinetics to nonlinear Michaelis-Menten kinetics. Exceedances in metabolic capacity result in toxicological manifestations (Argus et al., 1965, 1973; Kociba et al., 1974, 1975; NCI, 1978). A recent detailed study of the metabolism of 1,4-dioxane concludes that it is "likely that the true oncogenic substance in this case is dioxane itself, not a metabolic product", noting that "rodents demonstrate saturation of dioxane metabolism and develop neoplasms at concentrations above levels that saturate their metabolic capability (US Army, 2010). A study that gave numerous doses below the level of saturated metabolism, over 13-week duration in both rats and mice, did not demonstrate non-cancer effects at these doses (Kano et al., 2008).

Potentially Inconsistent Data

The mechanism by which 1,4-dioxane, or its metabolites are hepatotoxic has not been rigorously investigated. While liver toxicity due to uncharacterized metabolites cannot be ruled out (USEPA 2013), evidence suggests this is highly unlikely. Pretreatment with CYP 450 inducers did not enhance the toxicity of 1,4-dioxane.

KE#2 Sustained Cytotoxicity and Cell Death

Supporting Data (Strong/Moderate + 2)

Evidence of hepatocellular damage preceding tumor formation at higher doses of 1,4-Dioxane has been reported in multiple studies (Kociba et al., 1971, 1974). Liver changes including single cell necrosis at doses that saturate metabolism and depletion of cellular defenses have been reported as early as 11 weeks post 1,4-dioxane exposure (Stott et al., 1981; Kano et al., 2008; Kaisai et al., 2008, 2009). Stott et al. (1981) reported signs of liver cytotoxicity at 1000 mg/kg /day, but not at lower doses (10 mg/kg/day) following 11 weeks of exposure.

Potentially Inconsistent Data

Evidence in Kano 2009 study demonstrates liver tumor formation in mice without reported non-neoplastic changes, but this could be due to exceedance of the high dose necessary to saturate metabolism and lead to tumor formation and differences in pathological reporting of non-neoplastic changes across studies (Dourson et al., 2017). Dourson and colleagues note, "The reason that the findings in mice are not more supportive of the regenerative hyperplasia MOA, however, is because the histopathological characterizations of McConnell (2013) and of JBRC (1990a) in mice do not agree. McConnell (2013) found extensive liver noncancer toxicity as demonstrated by histopathology and fewer tumors than JBRC (1990a). JBRC (1990a) reported more tumors and nearly an absence of liver noncancer

histopathology in the chronic study. The lack of liver noncancer histopathology in JBRC (1990a) is unexpected, especially since an increase in liver enzymes associated with cell damage is found in this same study. Also, the JBRC (1990b) 13-week study showed extensive liver noncancer histopathology at suitably adjusted-to-chronic doses. Unfortunately, this internal inconsistency is not resolvable because slides or pictures from a sufficient number of experimental animals are not available for the current reanalysis."

KE#3 Regenerative Cellular Proliferation and Hyperplasia

Supporting Data (Moderate - +2)
Dose response and temporal data support the occurrence of cell proliferation and hyperplasia prior to the development of liver tumors in the rat model (Kociba et al., 1971, 1974). Using replicative DNA synthesis as a surrogate marker for cell proliferation, increased hepatocyte proliferation is typically reported at tumorigenic doses of 1,4 dioxane (Goldsworthy et al., 1991; Miyagawa et al., 1999; Stott et al., 1981; Uno et al., 1994). Cellular proliferation appears at 2 weeks (1.5- 2-fold increase) (Goldsworthy et al., 1991; Stott et al., 1981). Given sufficient sustained proliferation, preneoplastic foci development is initiated where histopathological changes are also reported (Kano et al., 2008; Lundberg et al., 1987; Kasai et al., (2008).

Potentially Inconsistent Data
No data to counter the appearance of cellular proliferations, but extant cell proliferation data (Goldsworthy et al., 1991; Miyagawa et al., 1999, Stott et al., 1981, Uno et al., 1994) provides no clear indications of cytotoxic or mitogenic responses for increased cell turnover. Kano et al., 2009 demonstrates liver tumor formation without reporting non-neoplastic key events. "Histopathological characterizations of McConnell (2013) and of JBRC (1990a) in mice do not agree. While McConnell (2013) found extensive liver noncancerous toxicity as demonstrated by histopathology and fewer tumors than JBRC (1990), JBRC reported more tumors and nearly an absence of any liver noncancerous histopathology in the chronic study which is highly unexpected, especially with an increase in liver enzymes associated with cell damage found in this same study" (Dourson et al., 2017). After extensive evaluation, Dourson et al. conclude that cytotoxicity and subsequent regenerative hyperplasia underpin the MOA.

KE#4 Preneoplastic Foci Development and Clonal Expansion of Initiated Cells (Moderate +2)

Supporting Data
Limited data provides evidence of liver foci development and clonal expansion. Study by Lundberg et al., 1987 evaluated tumor promotion ability of 1,4 dioxane after initiation and partial hepatectomy in terms of significant GGT-positive foci and lipid accumulation. Statistically increased preneoplastic foci volume was evident at 1000 mg/kg/day, the highest dose tested.

Potentially Inconsistent Data
Kano et al., 2009 demonstrates liver tumor formation without reporting non-neoplastic key events. "Histopathological characterizations of McConnell (2013) and of JBRC (1990a) in mice do not agree. While McConnell (2013) found extensive liver noncancerous toxicity as demonstrated by histopathology and fewer tumors than JBRC (1990), JBRC reported more tumors and nearly an absence of liver noncancerous histopathology in the chronic study which is unexpected especially with an increase in liver enzymes associated with cell damage found in this same study" (Dourson et al., 2017).

KE#5 Liver Tumors

Liver tumors evident in both longer- term, chronic cancer bioassays in rat and mice at high doses tested (Kano et al., 2009, Kociba, 1971, 1974 and NCI, 1978).

Table 2a. Incidence of Tumors or Adverse Health Outcome of Interest (Kano et al., 2009)

Tumor/adverse health outcome (Kano et al., 2009)	Rats							
	MALE				FEMALE			
	Control	11	55	274	Control	18	83	429
Adenoma	3	4	7	32	3	1	6	48
Carcinoma	0	0	0	14	0	0	0	10
Adenoma or Carcinoma	3	4	7	39	3	1	6	48
Kano et al., 2009	Mice							
	Control	49	191	677	Control	66	287	964
Adenoma	9	17	23	11	5	31	20	3
Carcinoma	15	20	23	36	0	6	30	45
Adenoma or Carcinoma	23	31	37	40	5	35	41	46

Table 2b. Incidence of Tumors or Adverse Health Outcome of Interest (Kociba et al., 1971, 1974)

Tumor/adverse health outcome (Kociba et al., 1974, 1971)	Rats							
	MALE				FEMALE			
	Control	9.6	94	1015	Control	19	148	1599
Adenoma or Carcinoma	1	0	0	6	0	0	1	4

Table 2c. Percent of Tumors or Adverse Health Outcome of Interest (NCI, 1978)

Tumor/adverse health outcome (NCI, 1978)	Rats							
	MALE				FEMALE			
	Control	240	530		Control	350	640	
Adenoma	6	3	3		0	30	34	
Carcinoma	0	3	0		0	0	3	
(McConnell, 2013, reread)	Mice[3]							
	Control	720	830		Control	380	860	
Adenoma	4.5	2	6		0	16	30	
Carcinoma	9	33	43		0	16	62	
Adenoma or Carcinoma	11	35	45		0	31	78	

Table 2d. Doses with Increased Incidence of Rodent Liver Adenomas and Carcinomas Across 2-year oral longer-term studies) (See Dourson et al, 2017 for dose response incidence figures and details)

Liver Tumor	Rats (Kano et al., 2009; Kociba et al., 1974 and NCI, 1978)										
	MALE (mg/kg/day)						FEMALE (mg/kg/day)				
	Control		274	550	1015		Control	429	640	1599	
Adenoma			+	+	+			+	+	+	
Carcinoma			+	+	+			+	+	+	
Adenoma or Carcinoma			+	+	+			+	+	+	
	Mice (Kano et al., 2009 and NCI 1978)										
	Control	49 (m)	191 (m)	677 (m)	720 (m)	830	66 (f)	287 (f)	964 (f)	380 (f)	860 (f)
Adenoma		-	+	+	+	+	+	+	+	+	+
Carcinoma		-	+	+	+	+	+	+	+	+	+
Adenoma or Carcinoma		-	+	+	+	+	+	+	+	+	+

[3] As indicated in Dourson et al., 2014, mice were treated at doses that saturate the metabolic capacity (KE#1) leading to an increased incidence of tumors.

Table 3a. Dose and Temporal Concordance Table (Oral Rat) – Extracted from Table 1 in Dourson 2017 – detailed references are provided in this paper.

Temporal	<13 weeks	11-13 weeks	>13 weeks	2-years	2-years
Male /Female Reported Doses (mg/kg/day)	KE#1 – Saturated metabolic capacity and presumed accumulation of proximal toxic moiety (parent compound)	KE#2 Sustained Cytotoxicity and cellular necrosis	KE#3 Regenerative cellular proliferation/hyperplasia	KE#4 Preneoplastic foci development and clonal expansion	KE#5 Liver Tumors
1000 (11 weeks) Stott et al., 1981 c	+	+	+	+	-
52/83 (13 weeks) Kano et al., 2008 & JBRC, 1990	-	-	-	-	-
126/185 (13 weeks) Kano et al., 2008 & JBRC, 1990	+	-	nd	-	-
274/427 (13 weeks) Kano et al., 2008 & JBRC, 1990	+	+	nd	-	-
657/756 (13 weeks) Kano et al., 2008 & JBRC, 1990	+	+ (females)	nd	-	-
1554/1614 (13 weeks) Kano et al., 2008 & JBRC, 1990	+	+	nd	-	-
11/18 (Kano 2yr) Kano et al., 2009 k & JBRC, 1990	-	-	nd	-	-
55/83 (Kano 2yr) Kano et al., 2009 k & JBRC, 1990	+	+ (males)	nd	+	-
274/429 (Kano 2yr) Kano et al., 2009 k & JBRC, 1990	+	+	nd	+	+
9.6/19 (Kociba 2yr) Kociba et al., 1974 & Kociba et al., 1971	-	+/- (males)	nd	-	-
94/148 (Kociba 2yr) Kociba et al., 1974 & Kociba et al., 1971	+	+	nd	-	-
1015/1078 (Kociba 2yr) Kociba et al., 1974 & Kociba et al., 1971	+	+	nd	+ (females)	+
240/350(NCI 2yr) NCI, 1978	+	nd	nd	+ (females)	+ (females)
550/640 (NCI 2yr) NCI, 1978	+	nd	nd	+	+ (females)

- + Observed
- - Not observed
- +/- Equivocal
- nd = not detected

[Note: see Dourson et al., 2014 and Dourson et al., 2017 for details on intensity of responses in relation to dose].

Table 3b. Dose and Temporal Concordance Table (Oral Mice) Extracted from Table 2 Dourson 2017
[Note: see Dourson et al., 2014 and Dourson et al., 2017 for details on intensity of responses in relation to dose]

- + Observed

Temporal	<13 weeks	13 weeks	>13 weeks	2-years	2-years
Male /Female Reported Doses (mg/kg/day)	KE#1 – Saturated metabolism leading to depletion of cellular defenses and presumed accumulation of proximal toxic moiety (parent compound)	KE#2 Sustained Cytotoxicity and cellular necrosis	KE#3 Regenerative cellular proliferation/hyperplasia	KE#4 Preneoplastic foci development and clonal expansion	KE#5 Liver Tumors
86/170 (13-wk) Kano et al., 2008 & JBRC, 1990 b	-	+/- (females)	nd	-	-
231/387 (13-wk) Kano et al., 2008& JBRC, 1990 b	-	+/- (females)	nd	-	-
585/898(13-wk) Kano et al., 2008 & JBRC, 1990 b	+	+ (weak = single cell necrosis, slight elevation serum AST ALT)	nd	-	-
882/1620(13-wk) Kano et al., 2008 & JBRC, 1990 b	+	+	nd	-	-
1570/2669 (13-wk) Kano et al., 2008 & JBRC, 1990 b	+	+	nd	-	-
49/66 (2 yr-Kano) Kano et al., 2009 & JBRC, 1990	-	-	nd	Unable to determine or not reported	+(female)
191/287(2 yr-Kano) Kano et al., 2009 & JBRC, 1990	+	+	nd	Unable to determine or not reported	+
677/964 (2 yr-Kano) Kano et al., 2009 & JBRC, 1990	+	+	nd	Unable to determine or not reported	+
380(female) (2yr NCI) NCI, 1978 and re-read	+	+ (males)	nd	+	+
720 (male) (2 yr NCI) NCI, 1978 and re-read	+	+	nd	+	+
830/860 (2yr NCI) NCI, 1978 and re-read	+	+	+	+	+

- - Not observed
- +/- Equivocal
- nd = not detected

B. Evolving Bradford Hill Causal Considerations: Qualitative and Quantitative Data Evaluation

Qualitative and quantitative rating categories. [See Becker et al. 2017 for details]

Qualitative	Quantitative	Category description
Strong	3	Multiple studies and/or extensive data provide convincing evidence that the substance causes the KE.
Moderate	2	Some evidence (direct or indirect) indicating the substance causes the KE, but scientific understanding is not yet completely established. There may be some studies that are equivocal.
Weak	1	Very limited evidence (direct or indirect) that the substance causes the KE along this pathway. Scientific understanding of the KE is limited.
No Evidence	0	No data available to support or negate causation of this KE by the substance.
Weak Counter	−1	There is very limited contradictory evidence (direct or indirect) that the substance does not cause this KE.
Moderate Counter	−2	Some evidence (direct or indirect) indicating that the KE is not caused by the substance, but scientific understanding is not completely established. There may be some studies that are equivocal.
Strong Counter	−3	Multiple studies and/or extensive data provide convincing evidence that the substance does not cause this KE.

Essentiality – *No data (Quantitative Rate = 0)*

Empirical Support (Dose and Incidence Concordance) – Overall, while there are some inconsistent data available to demonstrate dose and incidence concordance across the database, within each study, liver tumors are typically only formed at the highest dose tested in rats and across doses that exceed >200-300 mg/kg/day in the mouse, doses that exceed the metabolic capacity, deplete cellular defense mechanisms and presumed accumulation of toxic moiety (parent compound). Dourson et al. 2017 report that in rats, metabolic saturation occurs at 30 to 100 mg/kg, liver necrosis and/or inflammation at 219 mg/kg-day, increased DNA synthesis at 330 mg/kg-day, hyperplasia 330 mg/kg-day, foci development at 389 mg/kg-day and adenomas and carcinomas at 274-1015 mg/kg-day. In mice, Dourson et al report metabolic saturation occurs at 200 mg/kg, liver necrosis and/or inflammation at 190-200 mg/kg-day, increased hyperplasia at 380 mg/kg-day in one study but not the other study, foci development 380 mg/kg-day in one study but not the other study and adenomas and carcinomas at 66-1015 mg/kg-day.

Strong/Moderate rating (Quantitative Rate = 3) May be further strengthened by better understanding of the toxic moiety.*

Empirical Support (Temporal Concordance) – Overall, while there are some inconsistent data available to demonstrate temporal concordance of key events for the cytotoxic mode of action, liver tumor formation do not temporally precede non-neoplastic liver key events. Some liver tumors have been reported without the appearance of any earlier key events, which may be due to over saturation of metabolic capacity, as reported in the 2009 Kano cancer bioassay in mice, but this could also be due to differences in reporting non-apical endpoints as described in the Dourson et al., 2017.

Strong/Moderate rating (Quantitative Rate = 3)

Consistency – Consistent precursor key events are demonstrated across different studies, and the database presents a consistent demonstration of a non-genotoxic, non-linear mode of action (see comments in the characterization section). There are differences across the rat and mouse databases that presents some residual uncertainty.

Moderate rating (Quantitative Rate = 2)

Analogy – 1,4-Dioxane, a heterocyclic compound, is not structurally similar to other solvents such as chloroform or carbon tetrachloride, but within the functional category of solvents that operate via a sustained cytotoxic, persistent regenerative cellular proliferation mode of action, 1,4-dioxane exhibits very similar precursor key events leading to rodent liver tumor formation at high doses tested.

Strong (Quantitative Rate = 3)

C. Qualitative and Rating of the Key Events for Bradford Hill Causal Considerations

Table 4. Evolved Bradford Hill causal considerations, defining questions and body of evidence (adapted from Meek et al., 2014 a,b).

Bradford Hill Causal Considerations	Defining questions	Supporting Evidence	Potentially inconsistent Evidence	Weight of Evidence Scoring
Biological Plausibility	Does the hypothesized MOA conflict with broader knowledge? How well established is the MOA?	Well known and well established mode of action. 1,4-Dioxane operating via this pathway is strongly plausible.	Liver tumors have been demonstrated without the identified precursor key events, but this is largely driven by a single study.	Strong – not scored in this version of the methodology
Essentiality	Is the sequence of events reversible if dosing is stopped or a KE is prevented?	No data	Mouse Liver tumors are formed without demonstration of preneoplastic key events, but this is largely driven by a single study (see Dourson et al., 2017 for detailed discussion)	No data – 0
Empirical Support – Dose and Incidence Concordance	Dose concordance: Are the KEs observed at doses below or similar to those associated with the adverse effect? Incidence concordance: Is the occurrence of the adverse effect (e.g., hepatocellular carcinoma) less than that for the preceding KEs?	Precursor key events are observed at lower doses than tumorigenic doses, consistent within each study.	Tumors in mice were reported without non-neoplastic key events but this is largely driven by a single study (see Dourson et al., 2017 for detailed discussion).	Strong (+3)
Empirical Support – Temporal Concordance	Temporal concordance: Are the KEs observed in hypothesized order?	No tumors are formed before evidence of precursor key events.	Tumors in mice were reported without non-neoplastic key events but this is largely driven by a single study (see Dourson et al., 2017 for detailed discussion	Strong (+3)
Consistency	What is the pattern of observations across species/strains/organs/test systems? What would be expected based on the hypothesized MOA?	Rat data is consistent across different test systems.	Mice data is not entirely consistent (see Dourson et al., 2017 for detailed discussion	Moderate (+2)

Analogy	Would the MOA be anticipated based on broader chemical specific knowledge (e.g., the chemical is a member of a category for which related chemicals have known or strongly suspected MOA)?	Although not structurally related to 1,4-Dioxane, other solvents are similarly metabolized to more toxic metabolites and the cellular proliferation data shows sustained and persistent effects. Solvents such as chloroform and carbon tetrachloride operate via a cytotoxic mode of action.	No data provided	Strong (+3)

D. Quantification of the WOE for a Cytotoxic MOA

Table 5. Quantification of the WOE for the Cytotoxic MOA (Step 4 and 5)

Bradford Hill Causal Considerations	Key Event #1	Key Event #2	Key Event #3	Key Event #4	Key Event #5
	KE#1 – Saturated metabolism leading to depletion of cellular defenses	KE#2 Sustained Cytotoxicity and cellular necrosis	KE#3 Regenerative cellular proliferation/hyperplasia	KE#4 Preneoplastic foci development and clonal expansion	KE#5 Liver Tumors (time to tumor?)
Essentiality (40%)	0	0	0	0	0
Empirical Support – (20%) Dose and Incidence Concordance	.2(3) = 0.6	0.2(3) = 0.6	0.2(3) = 0.6	0.2(3) = 0.6	0.2(3) = 0.6
Empirical Support – (20%) Temporal Concordance	0.2(3) = 0.6	0.2(3) = 0.6	0.2(3) = 0.6	0.2(3) = 0.6	0.2(3) = 0.6
Consistency (10%)	0.1(2) = 0.2	0.1(2) = 0.2	0.1(2) = 0.2	0.1(2) = 0.2	0.1(2) = 0.2
Analogy (10%)	0.1(3) = 0.3	0.1(3) = 0.3	0.1(3) = 0.3	0.1(3) = 0.3	0.1(3) = 0.3
TOTAL	1.7	1.7	01.7 (0.1) = 0.17	1.7 (0.1) = 0.17	1.7 (0.1) = 0.17

Mode of Action Confidence Score = (3.91) ÷ (6.9) X 100 = 57 (Step 5)

E. Key References

Dourson M, Reichard J, Nance P, Burleigh-Flayer, H, Parker A, Vincent M, and McConnell EE. 2014. Mode of action analysis for liver tumors from oral 1,4-dioxane exposures and evidence-based dose response assessment. Regulatory Toxicology and Pharmacology 68 (2014) 387-401

Dourson M, Higginbotham J, Crum J, Burleigh-Flayer H, Nance P, Forsberg N, Lafranconi M, and Reichard. 2017. Update: Mode of Action (MOA) for Liver Tumors Induced by Oral Exposure to 1,4-Dioxane. Regulatory Toxicology and Pharmacology 88 (2017) 45-55.

US Army. 2010. Toxicology Report No, 87-XE-08WR-09, Studies on Metabolism of 1,4-Dioxane, March 2010. https://clu-in.org/download/contaminantfocus/dioxane/dioxane-tox-ada528633.pdf

V. Conclusion

Two hypothesized MOAs were evaluated for liver tumors induced in rodents by 1,4-dioxane using the quantitative MOA confidence scoring method described by Becker et al., 2017. This method provides a means to compare the WOE for the default MOA (induction of rodent liver tumors *via* a mutagenic MOA) to the WOE for a cytotoxicity and sustained regenerative cellular proliferation MOA.

The MOA confidence scoring results indicate it is highly unlikely that a mutagenic mode of action is plausible for 1,4-dioxane-induced rodent liver tumors. Based on the MOA confidence score of -45, the weight of evidence clearly does not support a mutagenic mode of action for 1,4-dioxane. The negative score indicates there is strong counter evidence for several of the early, diagnostic, KEs for a mutagenic MOA. In other words, the available data indicate that it is highly unlikely that rodent liver tumors are induced by 1,4-dioxane via a mutagenic mode of action.

In contrast, there are significant mechanistic data to support a non-linear, non-genotoxic mode of action for 1,4-dioxane. Based on the MOA confidence score of +57, the weight of evidence clearly supports a cytotoxic mode of action for 1,4-dioxane induction of rodent liver tumors. The MOA causal confidence scoring results indicate that the more likely operative MOA is cytotoxicity and regenerative cellular proliferation. Therefore, an increase in cancer risk would only occur at doses that exceed a specific threshold.

The overall pattern of observations is very consistent with a non-linear, threshold mode of carcinogenic action, as evident by the MOA confidence score of +57 for cytotoxicity compared to the mutagenic MOA score of -45. Therefore, it would be inappropriate to use a linear default for extrapolating cancer risks. Instead, the causal weight of the scientific evidence analysis supports use of a threshold, non-linear method for determining potential cancer risks.

VI. APPENDIX A. Genotoxicity Data Summary Tables (Extracted from IRIS Assessment for 1,4-Dioxane)

Table 4-16. Genotoxicity studies of 1,4-dioxane (in vitro)

Test system	Endpoint	Test conditions	Results[a]		Dose[b]	Source
			Without activation	With activation		
Prokaryotic organisms in vitro						
S. typhimurium strains TA98, TA100, TA1535, TA1537	Reverse mutation	Plate incorporation assay	–	–	10,000 µg/plate	Haworth et al. (1983, 028947)
S. typhimurium strains TA98, TA100, TA1530, TA1535, TA1537	Reverse mutation	Plate incorporation assay	–	–	ND	Khudoley et al. (1987, 194949)
S. typhimurium strains TA98, TA100, TA1535, TA1537	Reverse mutation	Plate incorporation and preincubation assays	–	–	5,000 µg/plate	Morita and Hayashi (1998, 195065)
Endpoint Test conditions Without activation With activation Dose[b] Source						
S. typhimurium strains TA100, TA1535	Reverse mutation	Preincubation assay	–	–	103 mg	Nestmann et al. (1984, 194339)
S. typhimurium strains TA98, TA100, TA1535, TA1537, TA1538	Reverse mutation	Plate incorporation assay	–	–	103 mg	Stott et al. (1981, 063021)
E. coli K-12 uvrB/recA	DNA repair	Host mediated assay	–	–	1,150 mmol/L	Hellmer and Bolcsfoldi (1992, 194717)
E. coli WP2/WP2uvrA	Reverse mutation	Plate incorporation and preincubation assays	–	–	5,000 µg/plate	Morita and Hayashi (1998, 195065)
P. phosphoreum M169	Mutagenicity, DNA damage	Mutatox assay	–	ND	ND	Kwan et al. (1990, 196078)
Nonmammalian eukaryotic organisms in vitro						
S. cerevisiae D61.M	Aneuploidy	Standard 16-hour incubation or cold-interruption regimen	–T	ND	4.75%	Zimmerman et al. (1985, 194343)
D. melanogaster	Meiotic nondisjunction	Oocytes were obtained for evaluation 24 and 48 hours after mating	+T[c]	ND[d]	2% in sucrose media	Munoz and Barnett (2002, 195066)

D. melanogaster	Sex-linked recessive lethal test	Exposure by feeding and injection	–	ND[d]	35,000 ppm in feed, 7 days or 50,000 ppm (5% in water) by injection	Yoon et al. (1985, 194373)
Mammalian cells in vitro						
Rat hepatocytes	DNA damage; single-strand breaks measured by alkaline elution	3-Hour exposure to isolated primary hepatocytes	+T[e]	ND[d]	0.3 mM	Sina et al. (1983, 007323)
Primary hepatocyte culture from male F344 rats	DNA repair	Autoradiography	–	ND[d]	1 mM	Goldsworthy et al. (1991, 062925)
L5178Y mouse lymphoma cells	Forward mutation assay	Thymidine kinase mutagenicity assay (trifluorothymidine resistance)	–	–	5,000 µg/mL	McGregor et al. (1991, 194381)

Test system	**Endpoint**	**Test conditions**	**Without activation**	**With activation**	**Dose[b]**	**Source**
L5178Y mouse lymphoma cells	Forward mutation assay	Thymidine kinase mutagenicity assay (trifluorothymidine resistance)	–	–T	5,000 µg/mL	Morita and Hayashi (1998, 195065)
BALB/3T3 cells	Cell transformation	48-Hour exposure followed by 4 weeks incubation; 13 day exposure followed by 2.5 weeks incubation	+T[f]	ND[d]	0.5 mg/mL	Sheu et al. (1988, 195078)
CHO cells	SCE	BrdU was added 2 hours after 1,4-dioxane addition; chemical treatment was 2 hours with S9 and 25 hours without S9	±[g]	–	10,520 µg/mL	Galloway et al. (1987, 007768)
CHO cells	Chromosomal aberration	Cells were harvested 8–12 hours or 18–26 hours after treatment (time of first mitosis)	–	–	10,520 µg/mL	Galloway et al. (1987, 007768)
CHO cells	SCE	3 hour pulse treatment; followed by continuous treatment of BrdU for 23 or 26 hours	–	–	5,000 µg/mL	Morita and Hayashi (1998, 195065)
CHO cells	Chromosomal aberration	5 hour pulse treatment, 20 hour pulse and continuous treatments, or 44 hour continuous treatment; cells were harvested 20 or 44 hours	–	–	5,000 µg/mL	Morita and Hayashi (1998, 195065)

		following exposure				
CHO cells	Micronucleus formation	5 hour pulse treatment or 44 hour continuous treatment; cells were harvested 42 hours following exposure	–	–	5,000 µg/mL	Morita and Hayashi (1998, 195065)
Calf thymus DNA	Covalent binding to DNA	Incubation with microsomes from 3-methylcholanthrene treated rats	–	–	0.04 pmol/mg DNA (bound)	Woo et al. (1977, 062950)

[a]+ = positive, ± = equivocal or weak positive, – = negative, T = toxicity. Endogenous metabolic activation is not applicable for in vivo studies.
[b]Lowest effective dose for positive results/highest dose tested for negative results; ND = no data.
[c]Rats were given doses of 0, 168, 840, 2,550, or 4,200 mg/kg at 4 and 21 hours prior to sacrifice. A 43 and 50% increase in the fraction of DNA eluted was observed for doses of 2,550 and 4,200 mg/kg, respectively. Alkaline elution of DNA was not significantly different from control in the two lowest dose groups (168 and 840 mg/kg).
[d]A dose-related increase in the incidence of bone marrow micronuclei was observed in male and female C57BL6 mice 24 or 48 hours after administration of 1,4-dioxane. A dose of 450 mg/kg produced no change relative to control, while doses of 900, 1,800, 3,600, and 5,000 mg/kg increased the incidence of bone marrow micronuclei by approximately two-,three-, four- and fourfold, respectively.
[e]A dose-related increase in the incidence of hepatocyte micronuclei was observed in partially hepatectomized mice 6 days after administration of 1,4-dioxane. A dose of 1,000 mg/kg produced no change relative to control, while doses of 2,000 and 3,000 mg/kg increased the incidence of hepatocyte micronuclei by 2.4- and 3.4-fold, respectively.
[f]Significant increases in the frequency of micronucleated erythrocytes were observed at each test dose of 1,4-dioxane (1,500, 2,500 and 3,500 mg/kg-day, 5 days/week).
[g]A dose-related increase in the frequency of micronuclei was observed in proliferating cells with micronuclei at 2,500 and 3,500 mg/kg-day, 5 days/week. No increase in the frequency of micronuclei was seen in the non-proliferating cells.
[h]No increase in the hepatocyte labeling index was observed 24 or 48 hours following a single gavage exposure of 1,000 mg/kg. Continuous administration of 1% 1,4-dioxane in the drinking water for up to 2 weeks produced a twofold increase in the hepatocyte labeling index.
[i]A similar pattern of RNA polymerase inhibition was observed at doses of 10 and 100 mg/rat. Inhibition was more pronounced at the higher dose.
[j]Hepatocyte viability was 86, 89, 87, 88, 78, and 86% 24 hours following exposure to 0, 1,000, 1,500, 2,000, or 4,000 mg/kg. The incidence (%) of replicative DNA synthesis was increased by 2.5-fold (1,000 mg/kg) or 4.5-fold (1,500 and 2,000 mg/kg). No increase in replicative DNA synthesis was observed at the highest dose (4,000 mg/kg).
[k]Replicative DNA synthesis was measured 24, 39, and 48 hours following a single dose of 0, 1,000, or 2,000 mg/kg. Hepatocyte viability ranged from 71 to 82%. The only increase in replicative DNA synthesis was observed 24 hours after administration of 2,000 mg/kg (threefold increase). Cell viability for this group was 79%.
[l]Replicative DNA synthesis was increased 1.5-fold in rats given 1,000 mg/kg of 1,4-dioxane for 11 weeks. No change from control was observed in rats exposed to 10 mg/kg for 11 weeks or rats acutely exposed to 10, 100, or 1,000 mg/kg.

Table 4-17. Genotoxicity studies of 1,4-dioxane

mammalian in vivo Test system	Endpoint	Test Conditions	Results[a]	Dose[b]	Source
Female Sprague Dawley Rat	DNA damage; single-strand breaks measured by alkaline elution	Two gavage doses given 21 and 4 hours prior to sacrifice	+[c]	2,550 mg/kg	Kitchin and Brown (1990, 062928)
Male Sprague Dawley Rat	DNA alkylation in hepatocytes	Gavage; DNA isolation and HPLC analysis	–	1,000 mg/kg	Stott et al. (1981, 063021)

		4 hours after dosing			
Male B6C3F1 Mouse	Micronucleus formation in bone marrow	i.p. injection; analysis of polychromatic erythrocytes 24 or 48 hours after dosing	–	Single dose of 4,000 mg/kg; 3 daily doses of 2,000	McFee et al. (1994, 195060)
Male and female C57BL6 Mouse; male BALB/c Mouse	Micronucleus formation in bone marrow	Gavage; analysis of polychromatic erythrocytes 24 or 48 hours after dosing	+ (C57BL6)[d] – (BALB/c)	900 mg/kg (C57BL6); 5,000 mg/kg (BALB/c)	Mirkova (1994, 195062)
Male CD1 Mouse	Micronucleus formation in peripheral blood	Two i.p. injections (1/day); micronucleated reticulocytes measured 24, 48, and 72 hours after the 2nd dose	–	3,200 mg/kg	Morita (1994, 196085)
Male CD1 Mouse	Micronucleus formation in hepatocytes	Gavage, partial hepatectomy 24 hours after dosing, hepatocytes analyzed 5 days after hepatectomy	+[e]	2,000 mg/kg	Morita and Hayashi (1998, 195065)
Male CD1 Mouse	Micronucleus formation in peripheral blood	Gavage, partial hepatectomy 24 hours after dosing, peripheral blood obtained from tail vein 24 hours after hepatectomy	–	3,000 mg/kg	Morita and Hayashi (1998, 195065)
Male CBA and C57BL6 Mouse	Micronucleus formation in bone marrow	Gavage; analysis of polychromatic erythrocytes from specimens prepared 24 hours after dosing	–	3,600 mg/kg	Tinwell and Ashby (1994, 195086)
Male CD1 Mouse	Micronuclei formation in bone marrow	Gavage; analysis for micronucleated erythrocytes 24 hours after dosing	+[f]	1,500 mg/kg-day for 5 days	Roy et al. (2005, 196094)
Male CD1	Micronuclei formation in	Gavage; analysis for	+[g]	2,500 mg/kg-day for 5 days	Roy et al.(2005, 196094)

Mouse	hepatocytes	micronuclei 24 hours after dosing			
Male Sprague Dawley Rat	DNA repair in hepatocytes	Drinking water; thymidine incorporation with hydroxyurea to repress normal DNA synthesis	–	1,000 mg/kg-day for 11 weeks	Stott et al. (1981, 063021)
Test system	**Endpoint**	**Test Conditions**	**Results[a]**	**Dose[b]**	**Source**
Male F344 Rat	DNA repair in hepatocytes (autoradiography)	Gavage and drinking water exposure; thymidine incorporation	–	1,000 mg/kg for 2 or 12 hours; 1,500 mg/kg-day for 2 weeks or 3,000 mg/kg-day for 1 week	Goldsworthy et al. (1991, 062925)
Male F344 Rat	DNA repair in nasal epithelial cells from the nasoturbinate or maxilloturbinate	Gavage and drinking water exposure; thymidine incorporation	–	1,500 mg/kg-day for 8 days + 1,000 mg/kg gavage dose 12 hours prior to sacrifice	Goldsworthy et al. (1991, 062925)
Male F344 Rat	Replicative DNA synthesis (i.e., cell proliferation) in hepatocytes	Gavage and drinking water exposure; thymidine incorporation	+[h] (1–2-week exposure)	1,000 mg/kg for 24 or 48 hours; 1,500 mg/kg-day for 1 or 2 weeks	Goldsworthy et al. (1991, 062925)
Male F344 Rat	Replicative DNA synthesis (i.e., cell proliferation) in nasal epithelial cells	Drinking water exposure; thymidine incorporation	–	1,500 mg/kg-day for 2 weeks	Goldsworthy et al. (1991, 062925)
Male Sprague Dawley Rat	RNA synthesis; inhibition of RNA polymerase A and B	i.v. injection; activity measured in isolated hepatocytes	+[i]	10 mg/rat	Kurl et al. (1981, 195054)
Male F344 Rat	DNA synthesis in hepatocytes	Gavage; thymidine and BrdU incorporation	+[j]	1,000 mg/kg	Miyagawa (1999, 195063)
Male F344 Rat	DNA synthesis in hepatocytes (replicative synthesis following toxicity)	Thymidine incorporation	±[k]	2,000 mg/kg	Uno et al. (1994, 194385)
Male Sprague Dawley Rat	DNA synthesis in hepatocytes (replicative synthesis following toxicity)	Drinking water; thymidine incorporation	+[l]	1,000 mg/kg-day for 11 weeks	Stott et al. (1981, 063021)

[a]+ = positive, ± = equivocal or weak positive, – = negative, T = toxicity. Endogenous metabolic activation is not applicable for in vivo studies.
[b]Lowest effective dose for positive results/highest dose tested for negative results; ND = no data.
[c]Rats were given doses of 0, 168, 840, 2,550, or 4,200 mg/kg at 4 and 21 hours prior to sacrifice. A 43 and 50% increase in the fraction of DNA eluted was observed for doses of 2,550 and 4,200 mg/kg, respectively. Alkaline elution of DNA was not significantly different from control in the two lowest dose groups (168 and 840 mg/kg).
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[f]Significant increases in the frequency of micronucleated erythrocytes were observed at each test dose of 1,4-dioxane (1,500, 2,500 and 3,500 mg/kg-day, 5 days/week).
[g]A dose-related increase in the frequency of micronuclei was observed in proliferating cells with micronuclei at 2,500 and 3,500 mg/kg-day, 5 days/week. No increase in the frequency of micronuclei was seen in the non-proliferating cells.

[h]No increase in the hepatocyte labeling index was observed 24 or 48 hours following a single gavage exposure of 1,000 mg/kg. Continuous administration of 1% 1,4-dioxane in the drinking water for up to 2 weeks produced a twofold increase in the hepatocyte labeling index.
[i]A similar pattern of RNA polymerase inhibition was observed at doses of 10 and 100 mg/rat. Inhibition was more pronounced at the higher dose.
[j]Hepatocyte viability was 86, 89, 87, 88, 78, and 86% 24 hours following exposure to 0, 1,000, 1,500, 2,000, or 4,000 mg/kg. The incidence (%) of replicative DNA synthesis was increased by 2.5-fold (1,000 mg/kg) or 4.5-fold (1,500 and 2,000 mg/kg). No increase in replicative DNA synthesis was observed at the highest dose (4,000 mg/kg).
[k]Replicative DNA synthesis was measured 24, 39, and 48 hours following a single dose of 0, 1,000, or 2,000 mg/kg. Hepatocyte viability ranged from 71 to 82%. The only increase in replicative DNA synthesis was observed 24 hours after administration of 2,000 mg/kg (threefold increase). Cell viability for this group was 79%.
[l]Replicative DNA synthesis was increased 1.5-fold in rats given 1,000 mg/kg of 1,4-dioxane for 11 weeks. No change from control was observed in rats exposed to 10 mg/kg for 11 weeks or rats acutely exposed to 10, 100, or 1,000 mg/kg.

VII. APPENDIX B. Quantitative Weight of Evidence to Assess Confidence in Potential Modes of Action (Becker et al., 2017)

Quantitative weight of evidence to assess confidence in potential modes of action

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ABSTRACT: The evolved World Health Organization/International Programme on Chemical Safety mode of action (MOA) framework provides a structure for evaluating evidence in pathways of causally linked key events (KE) leading to adverse health effects. Although employed globally, variability in use of the MOA framework has led to different interpretations of the sufficiency of evidence in support of hypothesized MOAs. A proof of concept extension of the MOA framework is proposed for scoring confidence in the supporting data to improve scientific justification for MOA use in characterizing hazards and selecting dose-response extrapolation methods for specific chemicals. This involves selecting hypothesized MOAs, and then, for each MOA, scoring the weight of evidence (WOE) in support of causality for each KE using evolved Bradford Hill causal considerations (biological plausibility, essentiality, dose-response concordance, consistency, and analogy). This early proof of concept method is demonstrated by comparing two potential MOAs (mutagenicity and peroxisome proliferator activated receptor-alpha) for clofibrate, a rodent liver carcinogen. Quantitative confidence scoring of hypothesized MOAs is shown to be useful in characterizing the likely operative MOA. To guide method refinement and future confidence scoring for a spectrum of MOAs, areas warranting further focus and lessons learned, including the need to incorporate a narrative discussion of the weights used in the evaluation and an overall evaluation of the plausibility of the outcome, are presented.